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Heat shock, high CO_2 and nitrogen fertilization effects in pepper plants submitted to elevated temperatures

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ABSTRACT

The atmospheric CO_2 concentration and the frequency and severity of heat waves are increasing, and the nitrogen - key to plant development - supplied to crops leaches easily, polluting ground water. In this experiment, these three elements were combined, in order to unravel the physiological mechanisms involved in the plant response to this future scenario.

Sweet pepper seedlings were exposed to distinct nitrate inputs (nutrient solutions containing 12 mM, 5 mM and 0 mM of N) and plant growth-promoting rhizobacteria, a high CO_2 concentration (1000 μmol mol^{-1}), and a heat shock (43 °C), in a controlled environment. Physiological markers - such as photosynthesis rate, stomatal conductance (gs), water use efficiency (WUE), chlorophylls, chlorophyll fluorescence, lipid peroxidation, anions, and free amino acids - were measured.

Exposure to the high temperature did not lead to any measurable stress in the sweet pepper plants. In fact, it augmented photosynthesis and the nitrate concentration, particularly at the elevated CO_2 concentration. Heat shock did not suppose any detriment for the plants beyond the expected increases in gs and WUE. On the contrary, heat triggered many processes that ended up favoring photosynthesis; when combined with elevated CO_2, the result was even more beneficial for the plants.

On the other hand, nitrogen starvation produced serious damage - such as decreases in the photosynthesis rate and chlorophylls and increases in lipid peroxidation and the levels of anions, cysteine, leucine, and phenylalanine in the plant - which was ameliorated to a great extent by the action of high CO_2. Finally, the plant growth-promoting rhizobacteria hardly enhanced plant growth and development, giving results similar to those of the nitrogen withdrawal treatment.

1. Introduction

Climate change is a serious threat to crops since they need to be edaphoclimatologically adapted to the place in which they are grown. This scenario is predicted to worsen since the atmospheric CO_2 concentration ($[CO_2]$) is expected to reach nearly 1000 μmol mol^{-1} by the end of the century (IPCC, 2013). Thus, many extreme climatic events have arisen as a consequence of the rise in the concentration of CO_2 and other exhaust gases. These climate-change events - droughts, flooding, high radiation, and salinity – are seriously affecting crops (Röth et al., 2016). In general, changes in $[CO_2]$ affect various physiological processes in plants, and previous studies have highlighted that high $[CO_2]$ increases photosynthetic carbon fixation rates, which stimulates plant growth and development. In addition, this responsiveness of plants to elevated CO_2 enables them to mitigate the effect of some of the above mentioned abiotic stresses, such as salinity (Geissler et al., 2010 and 2010, Piñero et al., 2014) or drought (Wall et al., 2006; Vu and Allen, 2009). Nevertheless, our understanding of the interactions among these factors remains incomplete (Pérez-López et al., 2013).

The increase in the $[CO_2]$ along with other greenhouse gases is expected to cause an increase in mean global temperature of more than 2 °C (IPCC, 2013). In addition to rising mean annual temperatures, the frequency, duration, and severity of heat waves will increase and might cause physiological disorders in plants and compromise their survival (Jiang and Huang, 2001). Therefore, although the heat-threshold level varies considerably among developmental stages, this global warming is an emerging and major challenge to sweet pepper (*Capsicum annuum* L.) growth and development and it seriously affects pollination and yield (Hedhly et al., 2009; Mateos et al., 2013). Protein stability can decrease under heat stress, exposing hydrophobic patches that cause the aggregation of denatured proteins (Kim and Hwang, 2015). The processes of sensing and responding to heat are complex phenomena in

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plants and comprise the activation of numerous regulatory and signaling pathways that eventually lead to a fine metabolic adjustment to ensure cell survival (Röth et al., 2016). Although high CO_2 and heat shock have been extensively studied, little is known about how their combination affects plants, or how these stress effects are influenced by the supply of nitrogen (N), another major pollutant.

Since N is one of the most important mineral nutrients for plant growth and its availability is considered one of the main limiting factors in crop production (Glass, 2003), N fertilizer was often used in excess in the past; as a consequence, N has produced considerable impacts in the environment, such as water contamination or soil imbalance. This problem is now being addressed by reducing fertilizer applications, to limit pollution. However, N is involved in the biosynthesis of many important compounds and the lack of it could cause a serious imbalance, resulting in reduced growth and lower yield of plants (Rubio-Wilhelmi et al., 2011). Actually, N affects many aspects of plants - including growth, photosynthesis, stomatal conductance, maximum potential quantum efficiency of photosystem II, and chlorophyll content. Therefore, a balance between proper plant growth and respect for the environment could be difficult to achieve. In the last decade, efficient N use under low N supply has been considered as one way to resolve these problems (Zhao et al., 2014). Moreover, plant-growth promoting rhizobacteria (PGPR) have been shown to fix atmospheric N and enhance water and mineral uptake, thereby allowing reduction of the mineral N supply and also partly mitigating several abiotic stresses (del Amor et al., 2008; Bashan and de-Bashan, 2010; del Amor and Cuadra-Crespo, 2012). Thus, PGPR could suppose a good alternative to traditional mineral nutrition, contributing to plant growth and avoiding pollution.

This study is the first attempt at comprehending how heat, CO_2, and N (bio-fertilizer and mineral) interact in a climate change scenario. It addresses the problem of N leaching in the future context of high [CO_2] and heat waves. In order to stimulate the physiological mechanisms affected by these three factors $-CO_2$, heat shock, and N -seedlings of sweet pepper were exposed to differing N inputs, high [CO_2], and a heat shock in a controlled environment. The level of stress produced was assessed by measuring the degree of change in the photosynthesis rate, stomatal conductance, water use efficiency, chlorophyll content, chlorophyll fluorescence, lipid peroxidation, anion concentration, and free amino acid concentration.

2. Material and methods

2.1. Plant material and growth conditions

Sweet pepper plants (≈ 10 cm and 10 leaves), cv. Herminio (Syngenta), were obtained from a commercial nursery 21 d after germination. They were grown in 5-l black containers filled with coconut coir fiber (Cocopeat, Pelemix). Sixty-four plants were used for the experiment and were irrigated with different modified Hoagland solutions (Table 1). Sixteen plants were irrigated with solution 1 (0 mM; no N-supply) as a control, 16 plants were also irrigated with solution 1 but a commercial PGPR product (*Azospirillum brasilense* strain M3 and *Pantoea dispersa* strain C3, immobilized in a solid support; Biopron®, Probelte) was added (following the manufacturer's instructions) as a source of N, 16 plants received solution 2 (5 mM) containing an intermediate N concentration (5 mM; low N-supply), and 16 plants received solution 3 (12 mM) with a complete supply of N (12 mM; full N-supply). The plants were drip-irrigated four times per day for 15 d and then eight times per day over the next 3 d (heat stress) in order to maintain a minimum of 35% drainage, to avoid salt accumulation (Piñero et al., 2016). The pH of the nutrient solutions was adjusted to 5.7, using 85% phosphoric acid, every 2 d. The plants were grown in a climate chamber designed by our department specifically for plant research purposes (del Amor et al., 2010), with fully-controlled environmental conditions: 50/70% RH, 16/8 h day/night, 24/20/28 °C (20/0/8 h) for 15 d, and 38/30/43 °C (20/0/8 h) for 3 d (heat stress) (Fig. 1). Photosynthetically active radiation (PAR) of 250 μmol m^{-2} s^{-1} was provided by a combination of fluorescent lamps (TL-D Master reflex 830 and 840, Koninklijke Philips Electronics N.V., Eindhoven, the Netherlands) and high-pressure sodium lamps (Son-T Agro, Philips). The experiment was performed at two [CO_2]: elevated (e[CO_2]), 1000 μmol mol^{-1}, or ambient (a[CO_2]), 400 μmol mol^{-1}. The [CO_2] was regulated by injection of external compressed air or CO_2 (bottle [CO_2] ≥ 99.9%), controlled by a Dräger Polytron IR CO_2.

Table 1
Nutrient composition of the different solutions used in the experiment.

Nutrient (per liter)	Solution 1	Solution 2	Solution 3
$MgSO_4 \cdot 7H_2O$ (mg)	602.37	556.22	494.91
KH_2PO_4 (mg)	408.38	291.26	135.65
$Ca(NO_3)_2 \cdot 4H_2O$ (mg)		606.50	
$CaSO_4$ - 0.02 N (ml)	549.88	250.92	1066.88
KNO_3 (mg)			295.81
K_2SO_4 (mg)	446.16	474.85	258.05
KOH - 1 N (ml)	0.43	0.31	0.14

2.2. Gas exchange

The net CO_2 assimilation rate (A_{CO2}), internal [CO_2] (Ci), transpiration rate (E), and stomatal conductance (gs) were measured in the youngest fully open leaf of each plant, using a CIRAS-2 (PP system, Amesbury, MA) with a PLC6 (U) Automatic Universal Leaf Cuvette, measuring both sides of the leaves. The cuvette provided light (LED) with a photon flux density of 1300 m^{-2} s^{-1}, a [CO_2] of 1000 or 400 μmol mol^{-1}, a leaf temperature of 28 or 45 °C, and 70% relative humidity. The water use efficiency (WUE) of leaf gas exchange was calculated from the gas exchange data as A/E, where A is the carbon assimilated through photosynthesis and E is the amount of water lost *via* transpiration.

2.3. Chlorophyll content and fluorescence

Chlorophylls *a* (Chl *a*), *b* (Chl *b*), and *a*+*b* (Chl *a* + *b*) were extracted from samples of the youngest fully open leaf with *N,N*-dimethylformamide, for 72 h, in darkness at 4 °C. Subsequently, the absorbance was measured in a spectrophotometer at 750, 664, and 647 nm, and the quantities were calculated according to the method of Porra et al. (1989).

On the leaf used for gas exchange, the dark-adapted maximum fluorescence (Fm) and minimum fluorescence (Fo) and the light-adapted, steady-state chlorophyll fluorescence (F) and maximum fluorescence (Fm′) were measured with a portable modulated fluorometer, model OS-30P (Opti-Science, USA). The ratio between the variable fluorescence from a dark-adapted leaf (Fv) and the maximal fluorescence from a dark-adapted, youngest fully open leaf (Fm) – called the maximum potential quantum efficiency of photosystem II (Fv/Fm) – was calculated. A special leaf clip holder was allocated to each leaf to maintain dark conditions for at least 30 min before reading.

2.4. Lipid peroxidation

Lipid peroxidation was measured as the amount of thiobarbituric acid-reactive substances (TBARS), as determined by the thiobarbituric acid (TBA) reaction (Heath and Packer, 1968). Lyophilized samples (0.1 g) were homogenized in 3 ml of 20% (w/v) trichloroacetic acid (TCA). The homogenate was centrifuged at 3500 × *g* for 20 min. To a 1.5-cm^3 aliquot of the supernatant, 1.5 cm^3 of 20% (w/v) TCA containing 0.5% (w/v) TBA and 0.15 cm^3 of 4% (w/v) BHT in ethanol were added. The mixture was heated at 95 °C for 30 min and then quickly cooled on ice. It was centrifuged at 10,000 × *g* for 15 min, then the absorbance was measured at 532 nm. The value for non-specific

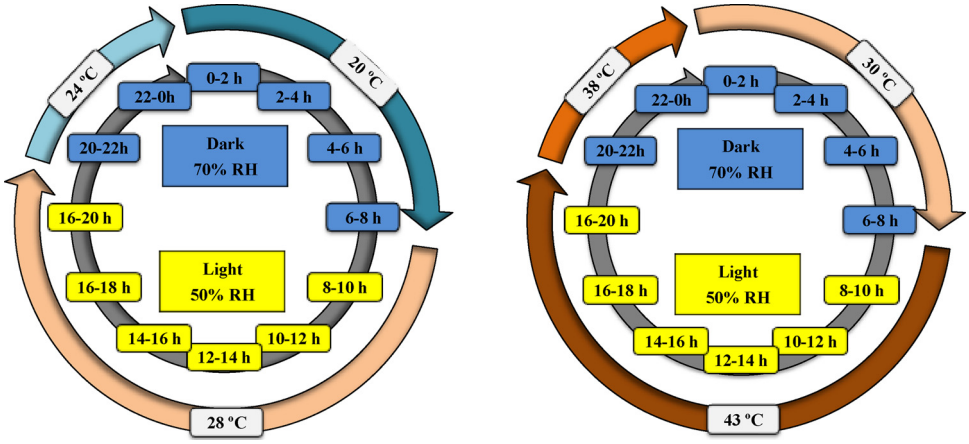

Fig. 1. Temperature (T[a]), relative humidity (RH) and light/dark conditions during the experiment, previous to heat shock (left) and during heat shock (right) at both CO_2 regimens.

absorption at 600 nm was subtracted. The concentration of TBARS was calculated using an extinction coefficient of 155 mM^{-1} cm^{-1} (Balestrasse et al., 2006).

2.5. Ion determination

Leaves were dried at 65 °C in a heater for 72 h. Anions were extracted from ground material (0.4 g) with 20 ml of deionized water. Samples were analyzed in an ion chromatograph (METROHM 861 Advanced Compact IC; METROHM 838 Advanced Sampler) and the column used was a METROHM Metrosep A Supp7 250/4.0 mm.

2.6. Free amino acids

Free amino acids were extracted from leaves frozen at 80 °C at the end of the experiment. After liquefying leaf samples, the sap was vortexed at 5000 rpm (10 min, 4 °C) and filtered (nylon membrane filter, 0.2 µm), and the free amino acids were determined by the AccQ Tag-Ultra Performance Liquid Chromatography (UPLC) method (Waters, 2006). For derivatization, 70 µl of borate buffer were added to the hydrolyzed sample or to 10 µl of the fruit sap. Following this, 20 µl of reagent solution were added. The reaction mixture was mixed immediately and heated at 55 °C for 10 min. After cooling, an aliquot of the reaction mixture was used for UPLC injection. The UPLC was performed on an ACQUITY UPLC system (Waters, Milford, MA, USA) equipped with a fluorescence detection (FLR) system. The column used was a BEH C18 100 mm × 2.1 mm × 1.7 µm (Waters, 2006). The flow rate was 0.7 ml min^{-1} and the column temperature was kept at 55 °C. The injection volume was 1 µl. The excitation (ex) and emission (em) wavelengths were set at 266 and 473 nm, respectively. The solvent system consisted of two eluents: (A) AccQ·Tag-ultra eluent A concentrate (5%, v/v) and water (95%, v/v); (B) AccQTag ultra eluent B. The following elution gradient was used: 0–0.54 min, 99.9% A–0.1% B; 5.74 min, 90.9% A–9.1% B; 7.74 min, 78.8% A–21.2% B; 8.04 min, 40.4% A–59.6% B; 8.05–8.64 min, 10% A–90% B; 8.73–10 min, 99.9% A–0.1% B. Empower 2 (Waters) software was used for system control and data acquisition. External standards (Thermo Scientific) were used for quantification of histidine, serine, arginine, glycine, aspartic acid, glutamic acid, threonine, alanine, proline, cysteine (cys), lysine, tyrosine, methionine, valine, isoleucine, leucine (leu), and phenylalanine (phe).

2.7. Statistical analysis

Twenty representative pepper plants were selected randomly for each treatment, namely, three N treatments in two CO_2 regimens. Six plants were measured; also, six samples were taken from each treatment for further analysis. The data were tested first for homogeneity of variance and normality of distribution. Significance was determined by analysis of variance (ANOVA), and the significance ($P < 0.05$) of differences between mean values was tested by Duncan's New Multiple Range Test, using Statgraphics Centurion® XVI (StatPoint Technologies Inc., The Plains, Virginia, USA).

3. Results

At the end of the experiment, the plants grown at e [CO_2] were markedly taller and more leafy than those cultivated at a[CO_2], as expected. However, the temperature did not affect the general appearance significantly, as determined visually, since no drought stress was applied along with the heat shock – in order to show the effects of heat alone on the pepper plants.

In the plants exposed to e[CO_2], the net rate of photosynthetic CO_2 assimilation was increased before and during the heat stress, when compared with the rates shown by the plants grown at a[CO_2] (Fig.2). The heat shock also induced an augmentation of the A_{CO2}, which was more intense at e[CO_2] than at a[CO_2]. This increase was influenced by the N fertilization at a[CO_2], since no significant differences were detected between the photosynthesis rates measured before and after the heat stress in the plants irrigated with 0 mM.

The decline in gs caused by e[CO_2] occurred at both temperatures in plants irrigated with 5 mM or 12 mM (no differences between the N treatments were detected at e[CO_2]) (Fig.2). However, this effect was only observed in plants supplemented with PGPR at the elevated temperature, and no decrease was observed in plants provided with any addition of N. The high temperature occasioned a rise in gs for all the plants except those supplied with 0 mM at a[CO_2].

The increase in the [CO_2] increased the WUE of the plants at both temperatures (Fig.2). The WUE declined at the elevated temperature, in both CO_2 regimens, as expected. The N treatment only affected the WUE at e[CO_2], where the most effective treatment was 5 mM, followed by 12 mM; no differences between plants supplemented with the biological fertilizer and those with no N addition were found.

The Chl *a* concentration was lower in both groups of plants grown without N in their irrigation solution (Fig.3). The levels of this chlorophyll were significantly lower in plants not supplied with biofertilizer at the high temperature, relative to 28 °C. No differences were found between temperatures or CO_2 treatments in the rest of the plants. The decrease in the abundance of Chl *b* in plants provided with the 0 mM solution (with and without biofertilization) was more marked than for Chl *a* (Fig.3). In terms of Chl *b*, no differences between temperatures were found. Nevertheless, it was more abundant at e[CO_2] when

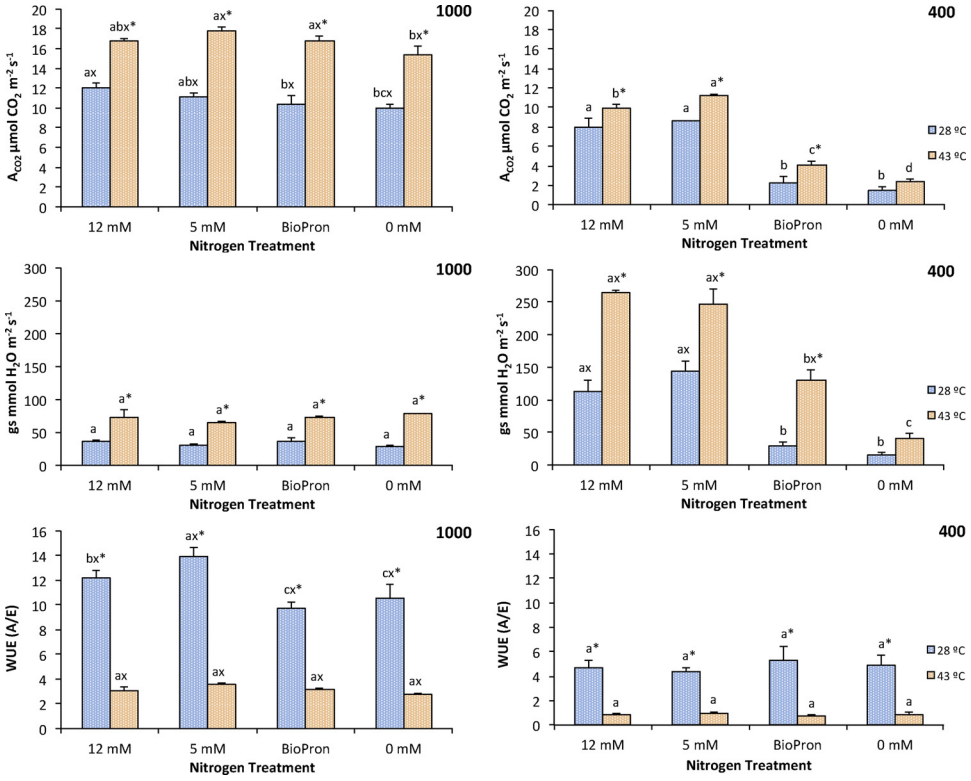

Fig. 2. Effects of heat shock and elevated CO_2 (1000 µmol mol^{-1}) on the net CO_2 assimilation rate (A_{CO2}), stomatal conductance (gs), and water use efficiency (WUE) in leaves of sweet pepper plants cv. 'Herminio' submitted to modified Hoagland solutions differing in their nitrate content (12 mM, 5 mM, 0 mM + PGPR, 0 mM) before (28 °C) and after heat shock (43 °C). Values are means ± SE. Letters indicate differences between nitrate treatments at the same temperature and CO_2; x denotes significant differences ($P < 0.005$) between CO_2 treatments for the same nitrate treatment and temperature; *denotes significant differences ($P < 0.005$) between plants submitted to the control temperature (28 °C) and those receiving a heat shock (43 °C) for the same CO_2 and nitrate treatments.

compared with plants grown at a[CO_2], in all N treatments except 0 mM. Differences identical to the ones observed for Chl *b* were found for Chl *a* + *b* (Fig. 3).

With regard to chlorophyll fluorescence, the withdrawal of mineral N from the nutrient solution provoked a decrease in the fluorescence detected from photosystem II of the chloroplasts (Fig. 3). The heat shock only induced a decrease in the Fv/Fm ratio in plants irrigated with the 0 mM solution, with or without PGPR, at a[CO_2]. A decrease in this ratio for the previously mentioned treatments was also produced at e[CO_2]. Nevertheless, the temperature did not have any effect at this [CO_2].

The amount of TBARS detected at e[CO_2] was significantly lower than at a[CO_2], in all N treatments (Fig. 4). At a[CO_2] the lipid peroxidation, evaluated as TBARS, gradually increased as the N supply decreased, regardless of the temperature. Contrasting with these findings, with respect to the temperature, are the results obtained at e[CO_2], in both the 0 mM and 0 mM + PGPR treatments, where the heat shock produced a decline in the TBARS concentration. For the cultivar used in this experiment, e[CO_2] produced a significant reduction in lipid peroxidation.

Chloride accumulation in leaves was markedly higher when mineral N was not supplied, at both [CO_2], and there was no effect of the PGPR (Fig. 5). The tissue nitrate concentration increased when the [CO_2] was high, even in those plants with no mineral N in their nutrient solution (Fig. 5). The heat shock also boosted nitrate accumulation in leaves, but only in the plants receiving mineral N. This higher amount was also detected in plants irrigated with 0 mM at e[CO_2]. However, the differences were not significant. It is noteworthy that the PGPR did not increase the leaf nitrate level.

The phosphate concentration was superior in the plants grown at a [CO_2] that received no N in their irrigation solution (Fig. 5). This concentration was even higher at 28 °C than at 43 °C. No differences were found at e[CO_2]. Sulfate was also more abundant in the plants with no N supply (including plants treated with biofertilizer) at a[CO_2] (Fig. 5). No differences among the N treatments were found at e[CO_2], at high temperature. However, plants grown with the 0 mM nutrient solution, even with the biofertilizer added, exhibited a lower leaf sulfate concentration at 28 °C.

Among all the amino acids studied in this experiment, significant differences were found only in cys, leu, and phe (non-significant differences are not shown). The concentration of cys was higher for both 0 mM treatments (0 mM and 0mM + PGPR) than for 12 mM at a[CO_2] (Fig. 6). At e[CO_2] cys also remained low in the 5 mM treatment. The high temperature decreased the level of cys in all treatments except for 12 mM at a[CO_2] and 5 mM at e[CO_2]. For leu and phe the response was identical at e[CO_2] and very similar at a[CO_2] (Fig. 6). For leu, significant differences were found between treatments 12 mM and 5 mM at 28 °C.

4. Discussion

In nature, heat stress usually coincides with water stress; these two stresses are often interconnected and produce similar cellular damage (Wang et al., 2003) - therefore, they are difficult to discern. However, under our experimental conditions, with well irrigated plants, heat stress promoted processes beneficial to plant growth and development. Traditionally, it has been considered that an increase in temperature means an increase in the photosynthesis rate, as long as the plant was previously maintained below its optimal temperature for development (Berry and Bjorkman, 1980). By contrast, if the plant was subjected to an increment in the surrounding air temperature, from the optimal to a supraoptimal temperature, the photosynthesis rate was reported to decrease (Hemantaranjan et al., 2014) due to limitations in either electron transport capacity or Rubisco activase capacity (Sage and Kubien, 2007). This is contrary to the findings of Rizhsky et al. (2002), who described no changes in photosynthesis along with augmentation of the stomatal conductance in response to high temperatures. It also contrasts with results in this experiment, where photosynthesis rate was augmented after heat shock. The optimal temperature range described for pepper growth is 20–30 °C (Li et al., 2015), since greater heat negatively affects the vegetative and reproductive growth phases of pepper (Kafizadeh et al., 2008). Therefore, and according to the present

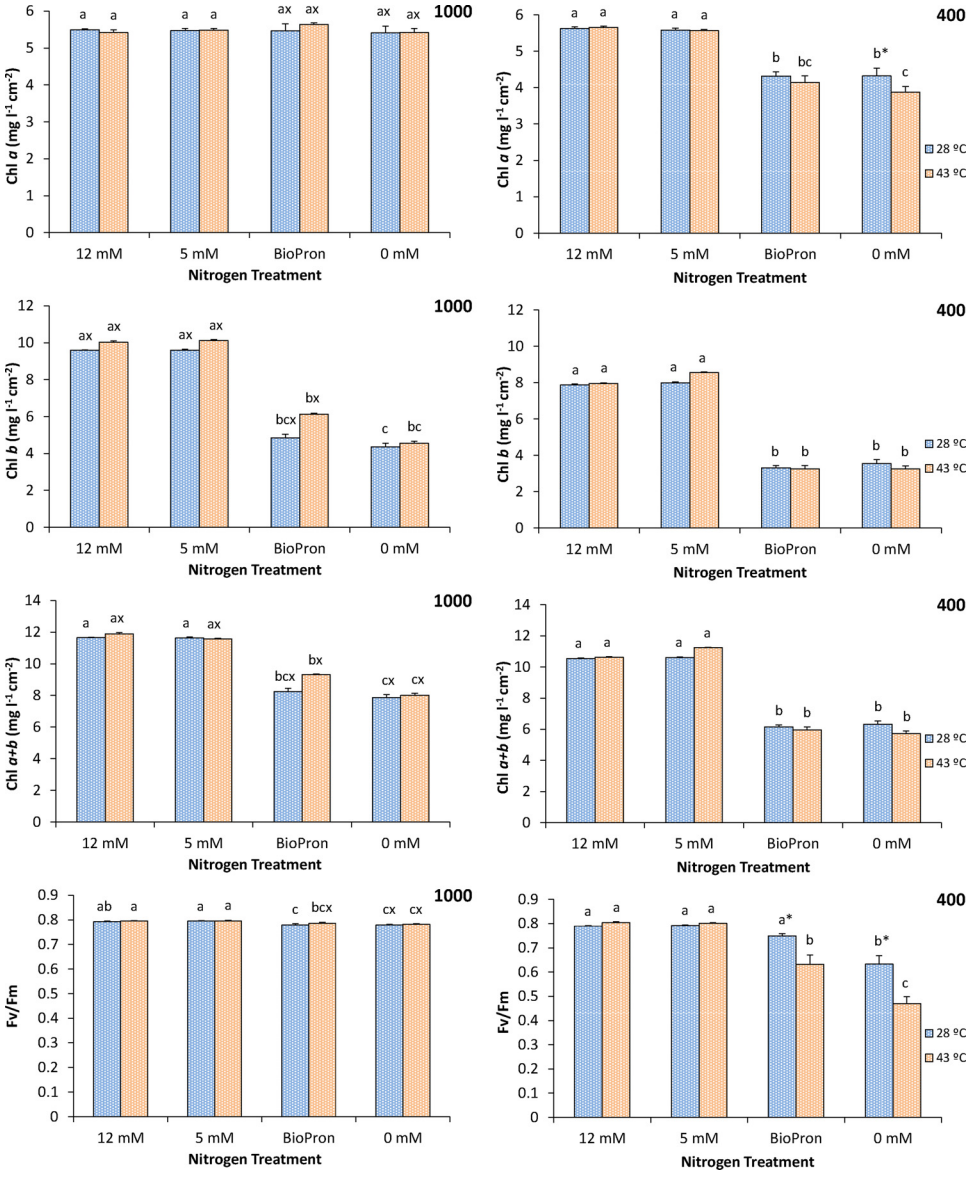

Fig. 3. Effects of heat shock and elevated CO_2 (1000 µmol mol^{-1}) on chlorophyll *a* (Chl *a*), chlorophyll *b* (Chl *b*), total chlorophylls (Chl *a* + *b*), and the maximum potential quantum efficiency of photosystem II (Fv/Fm) in leaves of sweet pepper plants cv. 'Herminio' submitted to modified Hoagland solutions differing in their nitrate content (12 mM, 5 mM, 0 mM + PGPR, 0 mM) before (28 °C) and after heat shock (43 °C). Values are means ± SE. Letters indicate differences between nitrate treatments at the same temperature and CO_2; x denotes significant differences ($P < 0.005$) between CO_2 treatments for the same nitrate treatment and temperature; *denotes significant differences ($P < 0.005$) between plants submitted to the control temperature (28 °C) and those receiving a heat shock (43 °C) for the same CO_2 and nitrate treatments.

study, the range in which growth is favored is wider than that in which sweet pepper is able to produce fruits, since temperatures above 32 °C can cause serious pollination and fertilization problems (Guo et al., 2014). Therefore, some damage assigned to heat could be due to its combination with water stress instead of heat shock alone.

The rates of many biological processes are increased by rising temperature due to effects on enzyme kinetics (Berry and Bjorkman, 1980), while many thermolabile compounds are cornerstones of the structure and metabolism of the plant (Hemantarajan et al., 2014). Thus, the heat tolerance shown by sweet pepper plants must be linked to heat response mechanisms, which might confer thermotolerance and protection on the photosynthetic apparatus, such that heat can stimulate the processes related to photosynthesis, while gene regulation takes place, correcting protein folding and preventing protein degradation (Obaid et al., 2016). Thus, to minimize the damage and ensure protection of cellular homeostasis, plants have evolved a complicated

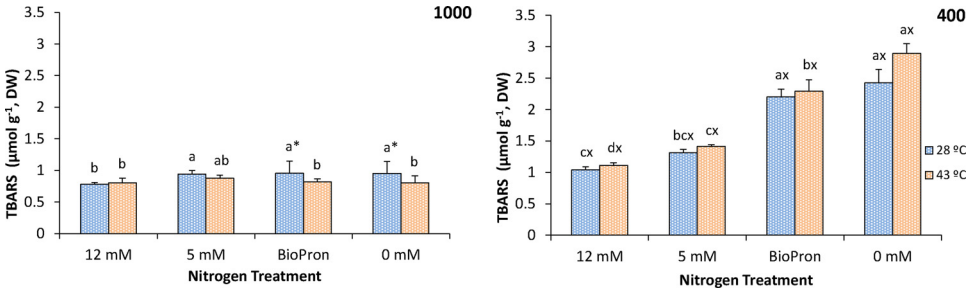

Fig. 4. Effects of heat shock and elevated CO_2 (1000 µmol mol^{-1}) on lipid peroxidation (TBARS) in leaves of sweet pepper plants cv. 'Herminio' submitted to modified Hoagland solutions differing in their nitrate content (12 mM, 5 mM, 0 mM + PGPR, 0 mM) before (28 °C) and after heat shock (43 °C). Values are means ± SE. Letters indicate differences between nitrate treatments at the same temperature and CO_2; x denotes significant differences ($P < 0.005$) between CO_2 treatments for the same nitrate treatment and temperature; *denotes significant differences ($P < 0.005$) between plants submitted to the control temperature (28 °C) and those receiving a heat shock (43 °C) for the same CO_2 and nitrate treatments.

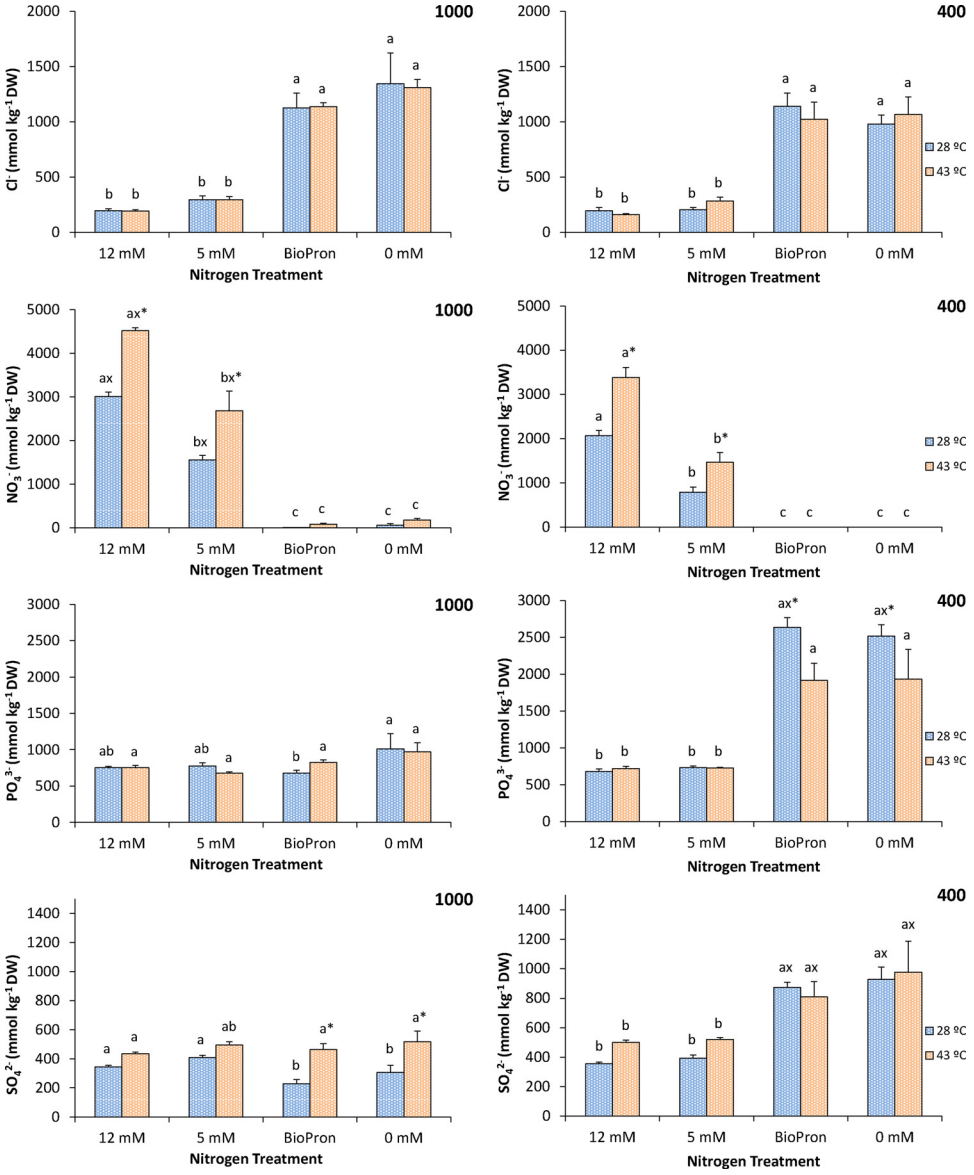

Fig. 5. Effects of heat shock and elevated CO_2 (1000 µmol mol^{-1}) on the chloride (Cl^-), nitrate (NO_3^-), phosphate (PO_4^{3-}), and sulfate (SO_4^{2-}) concentrations in leaves of sweet pepper plants cv. 'Herminio' submitted to modified Hoagland solutions differing in their nitrate content (12 mM, 5 mM, 0 mM + PGPR, 0 mM) before (28 °C) and after heat shock (43 °C). Values are means ± SE. Letters indicate differences between nitrate treatments at the same temperature and CO_2; x denotes significant differences ($P < 0.005$) between CO_2 treatments for the same nitrate treatment and temperature; *denotes significant differences ($P < 0.005$) between plants submitted to the control temperature (28 °C) and those receiving a heat shock (43 °C) for the same CO_2 and nitrate treatments.

response mechanism to deal with changes in environmental temperature (Li et al., 2015). These mechanisms involve the so called Heat Shock Proteins (HSPs), which interfere with the pathways by which heat damages plant physiology. These proteins are general stress proteins that protect cells from abiotic stress and facilitate recovery from heat-related damage (Wang et al., 2004). Therefore, heat shock did not affect the levels of chlorophylls, chloride, phosphate, or sulfate and produced no increase in lipid peroxidation in the sweet pepper plants receiving nitrate in their nutrient solution (5–12 mM N).

In contrast, N depletion, even with the addition of PGPR, caused serious disequilibrium in the sweet pepper plants at a[CO_2]; namely, A_{CO2}, gs, chlorophylls, fluorescence of the chloroplast, and nitrate decreased and lipid peroxidation, chloride, phosphate, and sulfate increased. Thus, the withdrawal of N implied either a worsening of the mechanisms of protection against heat damage activated by the plant or the malfunction of the normal processes of the plant due to deficiency. Regarding this aspect, Heckathorn et al. (1996) reported that the synthesis of HSPs requires N; therefore, a lack of N could be responsible for increased degradation of thermosensitive components. Nevertheless, although some parameters were more affected after heat shock, the majority of the malfunctions detected under N deprivation arose not only at high temperature, but also at 28 °C. Thus, the main part of the damage would be due directly to the absence of enough N to accomplish the many important roles which are normally played by N in plant metabolism (Shimoda and Maruyama, 2014), and the suppression of HSPs synthesis would be minimal.

The supply of e[CO_2] partially blocked the mechanisms of stress due to temporal N deprivation in sweet pepper plants. Thus, e[CO_2] mitigated parameters such as A_{CO2}, gs, Chl *a*, lipid peroxidation, and phosphate concentration. Despite this buffering action produced by e[CO_2], the descent detected in Fv/Fm, at both [CO_2], indicates damage in photosystem II of the chloroplast and explains the drop in Chl *b*, since large amounts of this pigment belong to this photosystem. This is in concordance with results reported by Zhang et al. (2013), who linked N application and Fv/Fm at e[CO_2]. On the other hand, the protective effect of e[CO_2] on Chl *a* probably was responsible for the preservation of the photosynthetic CO_2 assimilation rate in plants from the effect of N depletion at e[CO_2]. This finding is important because it partially contradicts the general idea that photosynthesis is restricted by the N availability in the plant (Iversen and Norby, 2008; Gutiérrez et al., 2013). It has been reported that an increase in the uptake of N leads to an enhanced photosynthetic response (Warren and Adams, 2002), as occurred in this experiment at a[CO_2]. However, this is not completely correct at e[CO_2], as reported by Aranjuelo et al. (2014), who detected

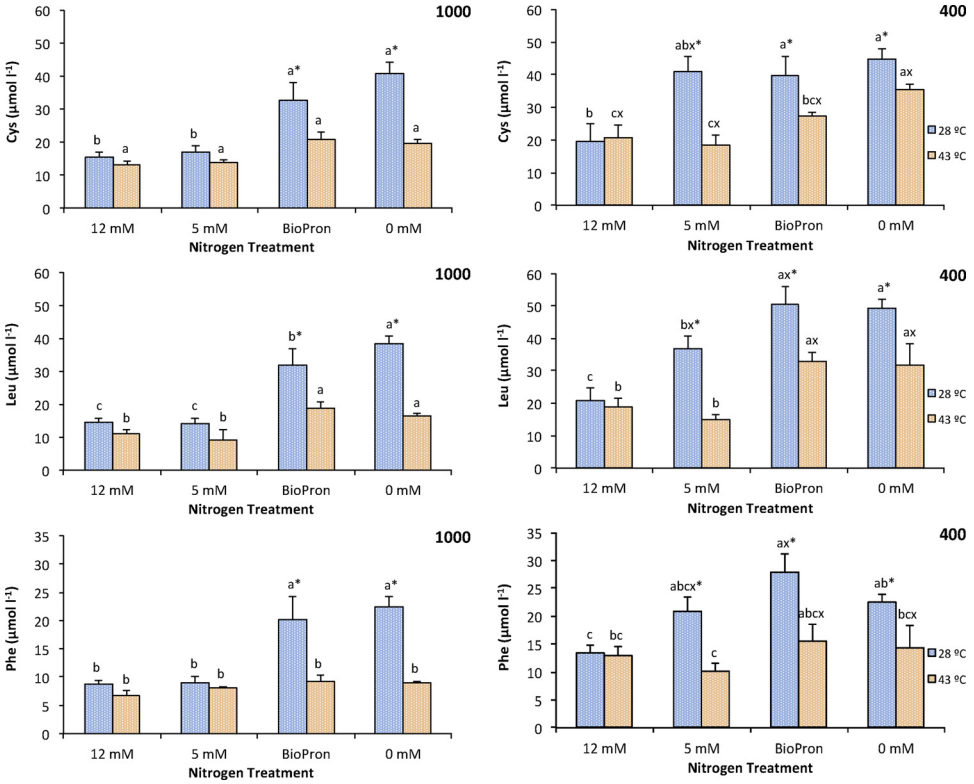

Fig. 6. Effects of heat shock and elevated CO_2 (1000 µmol mol^{-1}) on the cysteine (cys), leucine (leu), and phenylalanine (phe) concentrations in leaves of sweet pepper plants cv. 'Herminio' submitted to modified Hoagland solutions differing in their nitrate content (12 mM, 5 mM, 0 mM + PGPR, 0 mM) before (28 °C) and after heat shock (43 °C). Values are means ± SE. Letters indicate differences between nitrate treatments at the same temperature and CO_2; x denotes significant differences ($P < 0.005$) between CO_2 treatments for the same nitrate treatment and temperature; *denotes significant differences ($P < 0.005$) between plants submitted to the control temperature (28 °C) and those receiving a heat shock (43 °C) for the same CO_2 and nitrate treatments.

a higher A_{CO2} in pea plants grown at low N when the [CO_2] was elevated. Thus, e[CO_2] ameliorates the effects provoked by the lack of N in terms of gas exchange.

Notwithstanding, although e[CO_2] enhanced the WUE of the plants, N depletion significantly decreased the WUE at 28 °C, but only at e[CO_2], due to an increase in the transpiration of the plants at e[CO_2]. Thus, e[CO_2] made plants that received inadequate N fertilization more sensitive to drought. This loss of water was also detected in plants grown at high temperatures - at both [CO_2] - considering that heat increases evaporation and plant transpiration (Feller, 2006), contrary to what has been reported for e[CO_2] (Medlyn et al., 2001; Maherali et al., 2002; Ainsworth and Rogers, 2007; Gao et al., 2015). This interaction between heat and e[CO_2] leads plants to save water and raise their WUE, which supposes an advantage within a climate change context where periods of water shortage are expected to increase (Sreeharsha et al., 2015). This is because high temperatures affect photosynthesis by making it much more sensitive to e[CO_2] (Reich et al., 2016), which decreases also stomatal conductance and transpiration (Maherali et al., 2002).

The increasing level of N in the nutrient solution was directly related to the accumulation of nitrate in leaves. This accumulation was clearly stimulated by the high temperature, as well as by e[CO_2], as has been reported previously (Haynes and Goh, 1978; Lainé et al., 1993; Reich et al., 2016). The demand for N increases at elevated temperatures, to satisfy the increasing synthesis of HSPs, which makes the plant augment its nitrate uptake from the nutrient solution. On the other hand, e[CO_2] not only increased N uptake by the roots, but also would have remobilized nitrate from other parts of the plant, which would explain the presence of nitrate in leaves of plants irrigated with the 0 mM solution. The e[CO_2] also acted on sulfate and phosphate storage in leaves, mitigating the effect produced by the lack of N. Thus, N deficiency affects not only the processes in which N is involved, but also the uptake and accumulation of other anions. Thus, as has been reported for chloride (White and Broadley, 2001; Abdolzadeh et al., 2008; Song et al., 2009), the anions exhibited a drop in their uptake and content when the presence of nitrate augmented in the nutrient solution, due to the fact that most membrane anion channels are selective for nitrate over other anions such as chloride (Roberts, 2006). This augmentation of other anions in plants deprived of N was blocked by e[CO_2] for phosphate and sulfate, while heat shock did not interfere in chloride uptake or transport, and therefore did not induce deficiencies or toxicities in the plants.

The amino acids cys, leu, and phe were sensitive to high N demand and their concentrations decreased at high temperatures and when the concentration of nitrate in the nutrient solution was lower. Reich et al. (2016) did not find any differences in the free amino acids content related to temperature when different forms of N were applied, which indicates that N deficiency, directly or indirectly, is the promoter of these differences. Notwithstanding, Carfagna et al. (2011) found decreased cys levels in N-starved barley plants. However, the synthesis of cys appears to be limited to photosynthetic tissues (Howarth et al., 2008), where Carfagna et al. (2011) did not find any change after 21 days of N deprivation. Thus, the responses to N deprivation vary depending on the tissue and species.

In this experiment, high temperatures did not lead to any measurable stress in sweet pepper plants that were properly irrigated and fertilized. Actually, the high temperatures augmented photosynthesis and nitrate concentration, implying a better performance by the plants in terms of carbon fixation and storage, and hence growth (Hahn and Paek, 2001). All these beneficial effects on plant development were largely intensified by the action of e[CO_2], whose known effects were potentiated by heat. Needless to say, N starvation produced serious damage in the plants at a[CO_2], even when the plants were supplemented with PGPR - which, at least in this experiment, did not provide any benefit to the plants. By contrast, e[CO_2] was able to ameliorate the damage in N-starved plants, thereby making a difference to plant survival. Thus, e[CO_2] and heat action are two promoters of plant growth and development which, under climate change, might suppose also the avoidance of increasing drought and salinity stress and contamination of water reserves by N.

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